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Optimization of culture conditions for higher production of antimicrobial compounds by AZ-130 bacterial strain isolated from soil of Azerbaijan

A.G. Aghayeva

Institute of Molecular Biology and Biotechnologies, Azerbaijan National Academy of Sciences, 11 Izzat Nabiyev, Baku AZ1122, Azerbaijan; For correspondence: aytanaghayeva@gmail.com
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AZ-130 bacterial strain was isolated from soil sample collected from Azerbaijan in 2014. After preliminarily culture and supernatant screenings for novel antibacterial compounds, AZ-130 showed strong gram-positive activity against pathogenic *Staphylococcus aureus* and *Enterococcus faecalis* strains. Based on range of its activity, AZ-130 strain that produces AZ-130 antibacterial compound was selected for the further characterization. The main goal of this study is to optimize growth conditions for AZ-130 to determine the optimal medium, incubation temperature and time point in which the production of the antimicrobial compound is highest. To achieve this goal, 4 different media types at 4 different temperatures, in total 16 different growth conditions were tested. Supernatants were collected and clarified at day 1, 2 and 3 or 5. All collected supernatants were analyzed by spot test and broth microdilution method against *S.aureus*. According to the spot test and broth microdilution results, AZ-130 produces the most antimicrobial compound in TB + 2% Glucose medium at 32°C; incubation time is 2 days.

Keywords*: *Antimicrobial activity, antibiotics, bioactive molecules, culture conditions, medium optimization, natural products, primary metabolite, secondary metabolite, pathogenic bacteria

INTRODUCTION

The bacterial metabolism is a combination of all biochemical reactions occurring in a microbial cell lifelong. Metabolism ensures the reproduction of all cellular material and includes two opposite processes pathways - catabolism (destructive) and anabolism (constructive). Metabolites are the intermediates and products of metabolism. Depending on functional properties and metabolic pathways, metabolites classified into 2 groups - primary and secondary metabolites. Primary bacterial metabolites (nucleotides, amino acids, sugars, organic acids, vitamins) are low molecular weight compounds directly involved in growth, development or reproduction of the producing organism and present in every living cell. They serve as a primary source of energy for various biochemical and physiological functions necessary for the life and growth of the cell. Secondary bacterial metabolites (antimicrobial, antiparasitic and antitumor agents; enzymes and enzyme inhibitors etc.) are bioactive substances at low molecular weight that are in most cases not necessary for the life cycle of the producer. They play a role in the survival of producer organism in its ecosystem serving as poisons against competitors (Gokulan et al., 2014; Demain et al., 2000).

Antibiotics are one of the very important for human health secondary metabolites. Since 1928 - the year penicillin was discovered by A. Fleming - antibiotics have been the foremost weapon for combating such pathogenic microorganisms as *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* and others (Jones et al., 2017). However, during recent years the rapid emergence of antibiotic-resistant bacteria is occurring worldwide, and, unfortunately, nearly all available for treatment antibiotics are losing their effectiveness (Johnning et al., 2018). Bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* (CRAB), multidrug-resistant *Pseudomonas aeruginosa* (MDR) are becoming a critical threat to global health (Johnning et al., 2018; Lerminiaux et al., 2019;

Otto, 2013). Consequently, there is an urgent need to develop new, safe and effective antibiotics against bacterial infections. And considering that a majority of antibiotics used in the clinic today have been isolated from living organisms or are modified compounds which core structures derived from nature (Jones et al., 2017), it is very important to identify novel antibiotic producer strain.

Discovery and development of a new medicine, from target identification through approval for marketing is a very long and expensive process (Ekins, 2019). One of the main steps in antibiotics development is the optimization of growth conditions of the producer strain to maximize the final metabolite yield. Media components and their levels are crucial for the production of secondary metabolites. Even small changes in growth medium may affect not only the quantity of target-metabolite, but also the overall metabolic profile of producer strain. That is why before any large scale production of metabolites necessary to optimize growth condition of producer strain (Singh et al, 2017; Arul Jose et al., 2013; Wang et al., 2011).

The main objective of this study is to optimize growth conditions for AZ-130 bacterial strain to determine the optimal media, temperature and time point in which the production of the target AZ-130 antimicrobial compound is highest.

MATERIALS AND METHODS

To determine the optimal medium, temperature and time point in which the production of the AZ-130 compound is highest, 2 different media types (TSB - Tryptic Soy Broth and TB - Terrific Broth) +/- 2% Glucose at 4 different temperatures (18°C; 25°C; 32°C; 37°C) were tested (Table 1). To start all media with the same density of colonies, we made a preculture of AZ-130 strain and grown it at 32°C 250 RPM for 2 h. After 2 h, all 4 media (TB +/- Glucose; TSB +/- Glucose) were inoculated with 1mL of preculture of AZ-130 and grown at respective temperatures (18°C; 25°C; 32°C and 37°C). At time points of 24 h, 48 h and longer 1 mL of culture were collected. Bacteria-free culture supernatants were clarified by centrifugation at 10,000xg for 15 min at 4°C and filtering through 0.22 μm PES. Initially, all collected supernatants were tested for an inhibitory activity against *S.aureus* by the soft-agar overlay method as described by Hockett (Hockett et al., 2017) with some modifications. 10 μl of collected supernatants were spotted onto an agar plate of confluent indicator organisms. Plates were incubated at 37°C overnight and examined for the presence of inhibition zones in the place the supernatant has been spotted. The range of antibacterial activity was evaluated by measuring the diameter of the inhibition zone. Then, active in spot-test supernatants were analyzed by broth microdilution assay (Coyle et al., 2005). For broth microdilution assay 100 μl of supernatant was added to the first well of row and diluted 1:1 across the row. Then, 50 μL of *S.aureus* at required concentration, to obtain a final concentration of $5x10^4$ cells per well, was added into each well. As controls we used: positive 100% growth control – 50 μL of medium plus 50 μL of indicator organism; negative no cells control – 100 μL of medium. Microtiter plates were incubated at 37°C overnight in open biohazard bag (to hold moisture inside). After overnight incubation OD was read at 650 nm using Molecular Devices Spectra MaxPlus microplate reader. Data were analyzed and plotted.

Table 1. Components of TSB and TB media

Media componenets:	
TSB	**TB**
Casein peptone (pancreatic) - 17 g/L	Tryptone (pancreatic digest of casein) - 12 g/L
Dipotassium hydrogen phosphate - 2.5 g/L	Yeast extract 24 g/L
Glucose - 2.5 g/L	K_2HPO_4 9.4 g/L
Sodium chloride - 5 g/L	KH_2PO_4 2.2 g/L
Soya peptone (papain digest.) - 3 g/L	

RESULTS AND DISCUSSION

This research was carried out at Fraunhofer USA Center for Molecular Biotechnology. About 30 soil samples were collected from different areas of Azerbaijan during 2014-2018 years and sent to the Fraunhofer CMB for bacterial isolation and screening. A total of 578 bacterial strains were isolated from 30 soil samples and all of them were screened for antibacterial activity against two gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*) and two gram-negative (*Escherichia coli*) pathogenic strains by growth inhibition assay. After preliminarily culture screening 62 isolates showed an antibacterial activity against at least one pathogenic indicator organism. All 62 “active in culture” isolates were screened for an activity in cell-free culture supernatant. 14 isolates demonstrated an inhibitory activity in supernatant. They were categorized as “isolates of interest” and chosen for the further characterization. AZ-130 strain that produces antibacterial compound (AZ-130) is one of “isolates of interest”. It showed a strong antibacterial activity of 7 mm in supernatant against *S.aureus* during initial supernatant screening.

The effect of four different culture media at four different temperatures on bacterial growth was studied. Supernatants obtained from AZ-130 culture grown at respective temperatures were clarified and analyzed for an antibacterial activity against *S.aureus* by the growth inhibition assay (Fig. 1–Fig. 4).

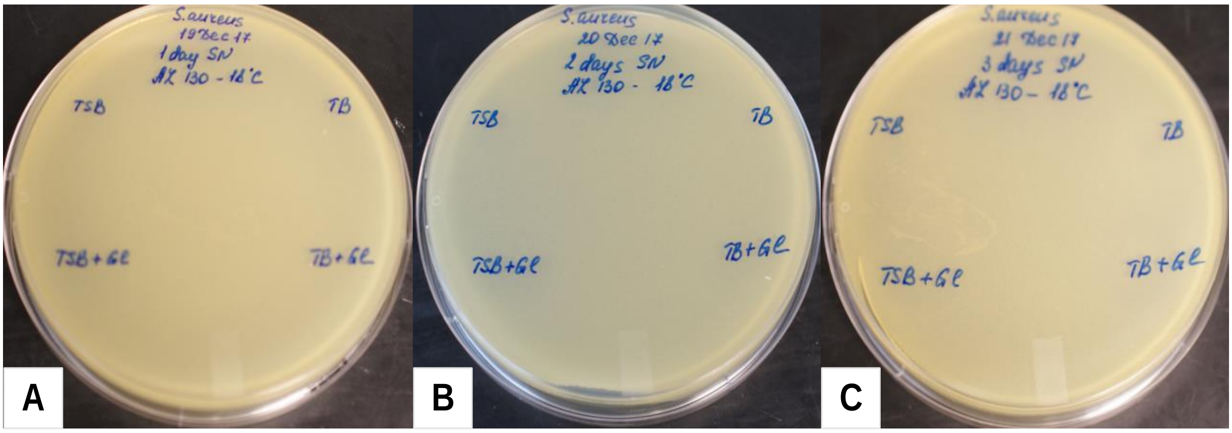


Fig. 1. Inhibitory effect of AZ-130 supernatants collected from cultures grown at 18°C against *S.aureus*. A) Supernatants collected at day 1. B) Supernatants collected at day 2. C) Supernatants collected at day 3.

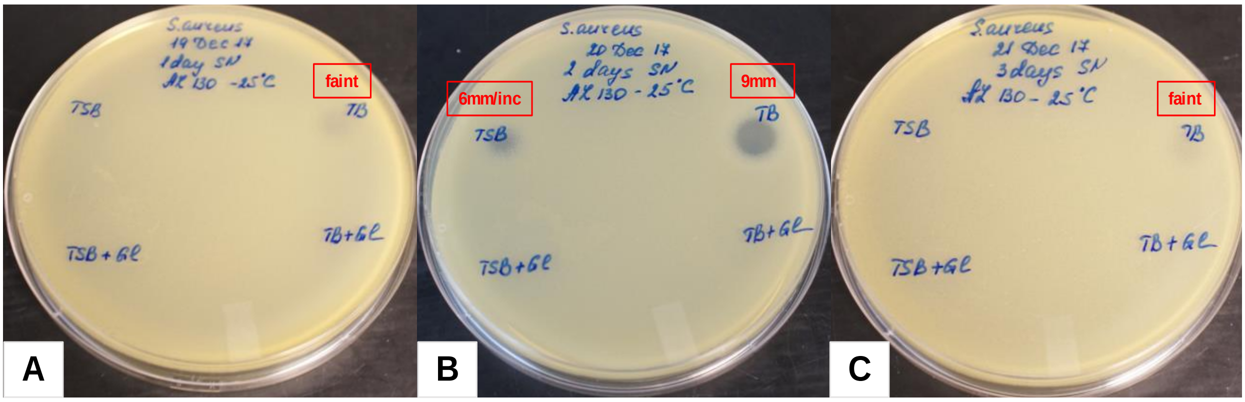


Fig. 2. Inhibitory effect of AZ-130 supernatants collected from cultures grown at 25°C against *S.aureus*. A) Supernatants collected at day 1. Only supernatant clarified from TB medium showed faint activity. B) Supernatants collected at day 2. Supernatants clarified from TSB and TB media showed respectively 6 mm/incomplete and 9 mm activity. C) Supernatants collected at day 3. Supernatant clarified from TB medium showed faint activity.

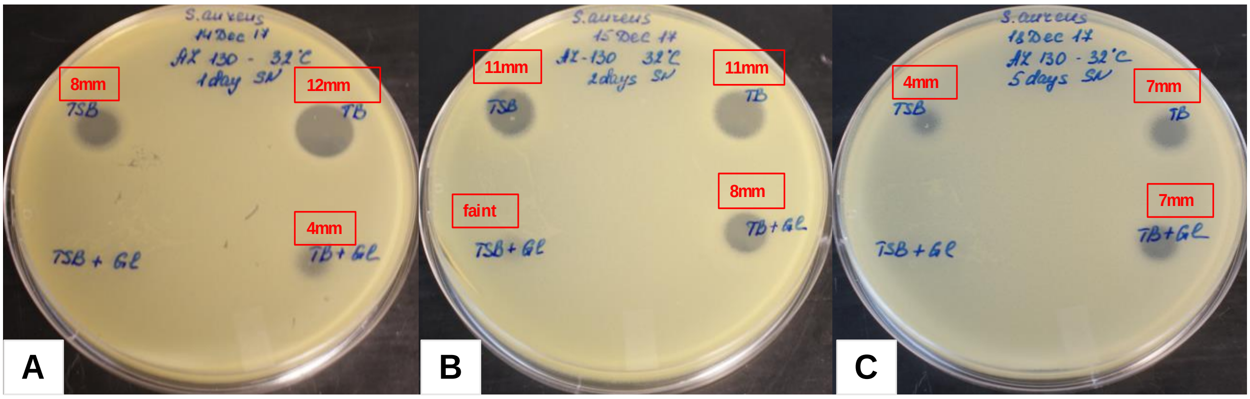


Fig. 3. Inhibitory effect of AZ-130 supernatants collected from cultures grown at 32°C against *S.aureus*. A) Supernatants collected at day 1. Supernatants clarified from TSB, TB and TB + 2% Glucose media showed respectively 8 mm, 12 mm and 4 mm activity. B) Supernatants collected at day 2. All clarified supernatants had an activity: TSB – 11 mm, TB – 11 mm, TSB + 2% Glucose – faint, TB + 2% Glucose – 8 mm. C) Supernatants collected at day 5. Supernatants clarified from TSB, TB and TB + 2% Glucose media showed respectively 4 mm, 7 mm and 7 mm activity.

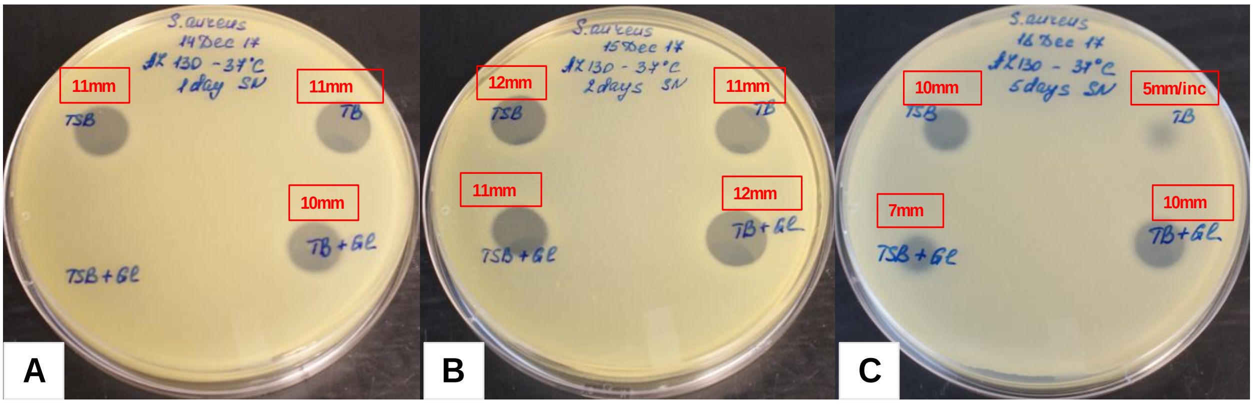


Fig. 4. Inhibitory effect of AZ-130 supernatants harvested from cultures grown at 37°C against *S.aureus*. A) Supernatants collected at day 1. Supernatants clarified from TSB, TB and TB+2% Glucose media showed respectively 11 mm, 11 mm and 10 mm activity. B) Supernatants collected at day 2. All clarified supernatants had an activity: TSB – 12 mm, TB – 11 mm, TSB + 2% Glucose – 11 mm, TB + 2% Glucose – 12 mm. C) Supernatants collected at day 5. All clarified supernatants had an activity: TSB – 10 mm, TB – 5 mm/incomplete, TSB + 2% Glucose – 7 mm, TB + 2% Glucose – 10 mm.

As can be seen from the Fig. 1 AZ-130 didn't produce any antimicrobial compound with gram-positive activity when it grown at 18°C; all supernatants collected at day 1, 2 and 3 didn't show any activity. In three other tested temperatures production of antimicrobial compound depends on media components and incubation time (Fig. 2-Fig. 4).

The production of AZ-130 compound by AZ-130 strain grown in TSB medium rises as the incubation time and temperature goes up (ZOI at day 1: 25°C – none, 32°C – 8 mm, 37°C – 11 mm; ZOI at day 2: 25°C – 6 mm/incomplete, 32°C – 11 mm and 37°C – 12 mm), before declining at day 3 or 5 (ZOI at 25°C – none, 32°C – 4 mm and 37°C – 10 mm).

The secretion of AZ-130 compound by AZ-130 strain grown in TB medium also fluctuates depending on growth temperature. Faint activity observed at day 1 from culture grown at 25°C sharply increases to strong 11-12 mm activity at 32°C and 37°C. The same upward trend in supernatants activities was observed at day 2 (ZOI at 25°C – 9 mm, at 32°C – 11 mm, at 37°C – 11 mm). AZ-130 partially loses its activity at day 3 or 5 (ZOI at 25°C – faint, 32°C – 7 mm, 37°C – 5 mm/incomplete).

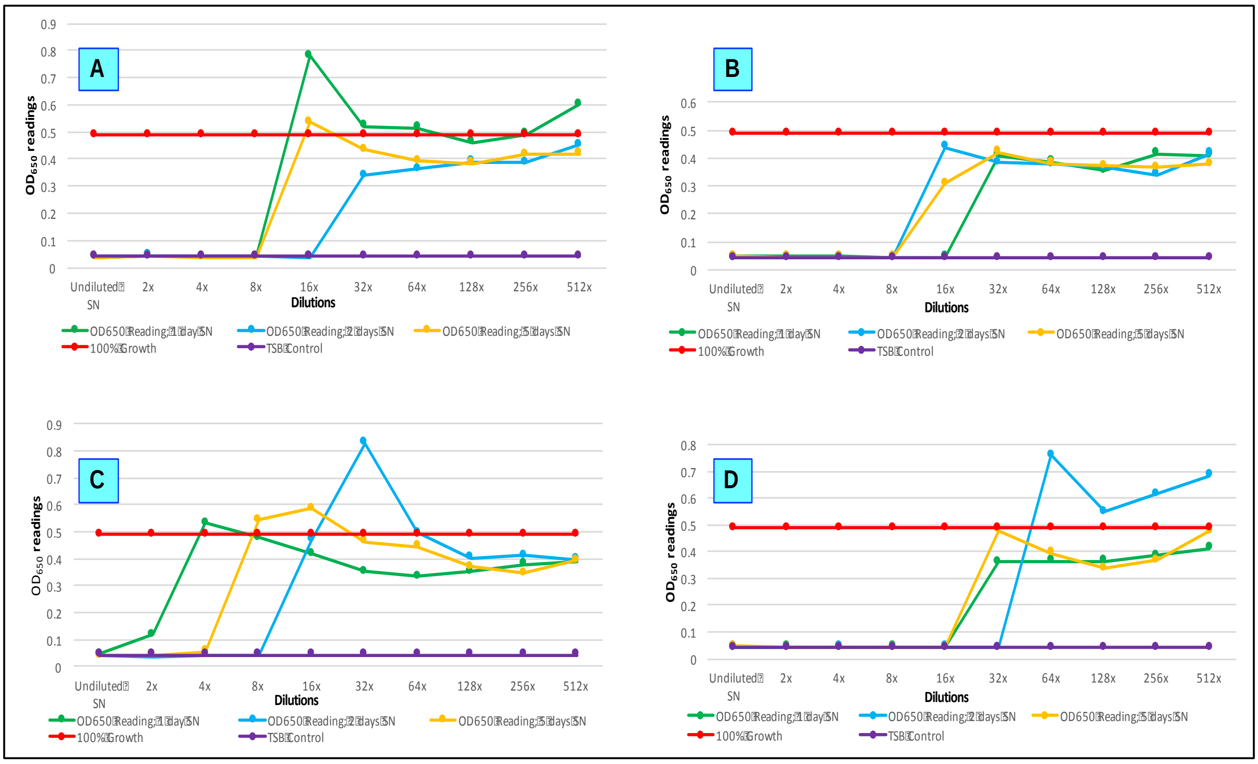


Fig. 5. Growth kinetics of *S.aureus* in the presence of AZ-130 supernatants (32°C) clarified from: A) TSB medium; B) TB medium; C) TSB + 2% Glucose; D) TB + 2% Glucose.

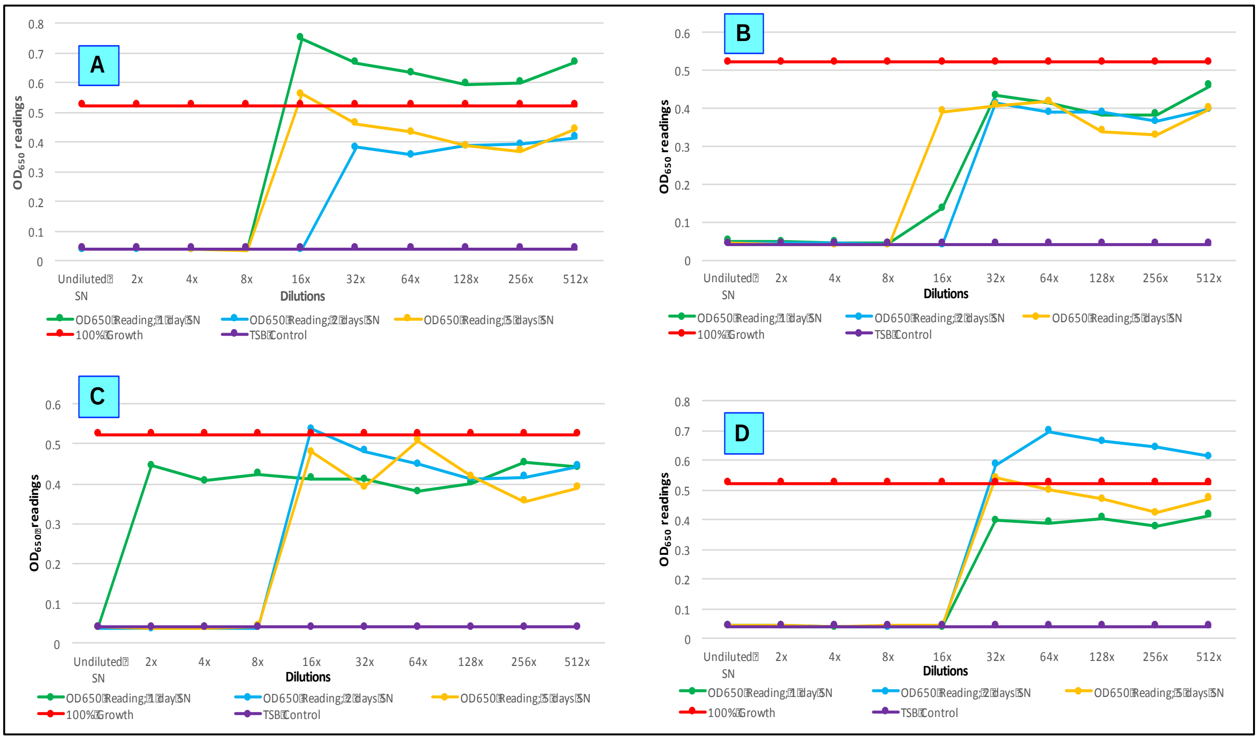


Fig. 6. Growth kinetics of *S.aureus* in the presence of AZ-130 supernatants (37°C) clarified from: A) TSB medium; B) TB medium; C) TSB + 2% Glucose; D) TB + 2% Glucose.

AZ-130 strain grown in TSB + 2% Glucose medium doesn't produce enough compound at day 1, however, at day 2 the concentration of compound grows as the temperature rises (ZOI at 25°C – no activity, 32°C – faint, 37°C – 11 mm) and decreases again at day 3 or 5 (ZOI at 25°C - none, 32°C – none and 37°C – 7 mm).

In terms of TB + 2% Glucose medium AZ-130 strain produces an antimicrobial compound when it grown at 32°C and 37°C. The activity of AZ-130 increases from 4 mm at day 1 to 8 mm at day 2 and slightly drops to 7 mm at day 5 (32ºC). Similar tendency in activity of AZ-130 observes at 37°C (ZOI at day 1 – 10 mm, day 2 – 12 mm and day 5 – 10 mm).

To summarize spot-test results, AZ-130 strain produces the most antimicrobial compound when it grown at 32°C and 37°C. We didn't observe any activity in supernatants collected from AZ-130 cultures grown at 18°C. We observed some activity in supernatants collected from AZ-130 cultures grown at 25°C, but those activities weren't as strong as they were in supernatants collected from cultures grown at 32°C and 37°C. In the next step, to be able to compare the concentration of bactericidal units in active supernatants, collected from cultures grown at 32°C and 37°C, they were analyzed by broth microdilution assay. Results of broth microdilution assay are presented in the Fig. 5 and Fig. 6.

As we see from the Fig. 5 in three out of four tested media at 32°C the highest production of AZ-130 observes at day 2. Supernatant collected from TSB + 2% Glucose active after eight-fold, from TSB after sixteen-fold and from TB+2% Glucose after thirty-two-fold dilution. Supernatant collected from TB medium shows its maximum activity of sixteen-fold at day 1.

From the Fig. 6 (growth of AZ-130 at 37°C) it's clear that the activity of sixteen-fold diluted AZ-130 compound collected from TB + 2% Glucose is stable even after five days of incubation. Supernatants of AZ-130 collected from TB and TSB media showed maximum activity of sixteen-fold at day 2; supernatants collected from TSB + 2% Glucose showed maximum activity of eight-fold at day 2 and day 5.

In summary, AZ-130 produces the most antibacterial compound when it grown in TB+2% Glucose medium at 32°C for 2 days. Observed activities are strong and stable, since AZ-130 biomolecule doesn't lose activity even after 5 days of incubation.

Summarized results of spot-test and broth microdilution assay are given in the Table 2.

Table 2. Summary of spot test and broth microdilution assay results.

Temperature	**Medium**	**Agar spot test results ZOI, mm**			**Broth Microdilution results Active supernatants' dilution**		
		SN at day 1	**SN at day 2**	**SN at day 3 / day 5**	**SN at day 1**	**SN at day 2**	**SN at day 5**
18°C	**TSB**	none	none	none			
	TB	none	none	none			
	TSB+2 % Glucose	none	none	none			
	TB +2 % Glucose	none	none	none			
25°C	**TSB**	none	6 /inc	none			
	TB	faint	9	faint			
	TSB + 2% Glucose	none	none	none			
	TB + 2% Glucose	none	none	none			
32°C	**TSB**	8	11	4	*8*	*16*	*8*
	TB	12	11	7	*16*	*8*	*8*
	TSB + 2% Glucose	none	faint	none	*undiluted*	*8*	*4*
	TB + 2% Glucose	4	8	7	**16**	**32**	**16**
37°C	**TSB**	11	12	10	*8*	*16*	*8*
	TB	11	11	5 /inc	*8*	*16*	*8*
	TSB + 2% Glucose	none	11	7	*undiluted*	*8*	*8*
	TB + 2% Glucose	10	12	10	**16**	**16**	**16**

CONCLUSION

Discovery and development of a new antimicrobial compound, from target identification through approval for marketing, is a very long and expensive process (Ekins S., 2019). One of the main steps in antibiotics development is the optimization of growth conditions of the producer strain to maximize the final metabolite yield (Singh et al, 2017; Arul Jose et al., 2013; Wang et al., 2011).

The results obtained fully confirm the importance of optimizing even the most insignificant parameters of growing medium. Temperature, incubation time, media components play significant role in the production of AZ-130 biomolecule by AZ-130 bacterial strain.

According to the spot-testing and 96-well plate microdilution assay results, AZ-130 strain produces the most antimicrobial compound in TB + 2% Glucose at 32°C; incubation time is 2 days. The production of AZ-130 compound in this medium is at least twofold higher in compare with all other tested conditions.

Further characterization of AZ-130 compound will involve the isolation/purification of secreted bioactive compound from cell-free culture supernatant, determination of MIC values against pathogenic lab and clinical strains, determination the chemical structure of novel biomolecule and confirmation the antimicrobial activity in vivo.

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Azərbaycan torpaqlarından ayrılmış AZ-130 bakteriya ştamının yüksək miqdarda antibakterial birləşmənin sintezi üçün böyümə şəraitinin optimallaşdırılması

A.Q. Ağayeva

AMEA Molekulyar Biologiya və Biotexnologiyalar İnstitutu

2014-cü ildə Azərbaycandan toplanmış torpaq nümunəsindən AZ-130 ştamı ayrılmışdır. Yeni antibakterial birləşmələrin aşkarlanması üçün aparılmış ilkin kultura və supernatant skrininqinin nəticəsində AZ-130 ştamı *Staphylococcus aureus* və *Enterococcus faecalis* qram-müsbət patogenlərə qarşı güclü fəallıq göstərmişdir. Onun fəallığını nəzərə alaraq, AZ-130 antibakterial birləşməni istehsal edən AZ-130 ştamı xüsusiyyətlərinin daha dərin öyrənilməsi üçün seçilmişdir. Aparılmış tədqiqat işinin məqsədi antimikrob birləşmənin istehsalının ən yüksək olduğu optimal mühit, inkubasiya temperaturu və zaman nöqtəsini müəyyənləşdirmək üçün AZ-130 ştamının böyümə şəraitinin optimallaşdırılmasıdır. Bu məqsədə, 16 fərqli böyümə şəraiti (4 fərqli temperaturda 4 fərqli mühit növü) yoxlanılmışdır. Supernatantlar 1-ci, 2-ci və 3/5-ci günlərdə toplanaraq təmizlənmişdir. Toplanan bütün supernatantlar bakteriyanın böyüməsinin inhibə edilməsi və mikrodurulaşdırma metodları ilə təhlil edilmişdir. Əldə edilmiş nəticələrə əsasən, AZ-130 biomolekulunun ən yüksək istehsalı AZ-130 ştamının 32°C-də TB + 2% glükoza mühitində müşahidə edilir.

Açar sözlər: *Antimikrob fəallıq, antibiotiklər, bioaktiv molekullar, kultural mühit, böyümə mühitinin optimallaşdırılması, təbii məhsullar, ilkin metabolit, ikinci metabolit, patogen bakteriyalar*

Оптимизация условий культивирования для более высокой продукции антимикробных соединений бактериальным штаммом AZ-130, выделенным из почвы Азербайджана

А.Г. Агаева

Институт молекулярной биологии и биотехнологий НАН Азербайджана

Штамм AZ-130 был изолирован из образца почвы, отобранного в Азербайджане в 2014 году. После предварительного скрининга на наличие антимикробной активности в культуре и в супернатанте, AZ-130 показал сильную грамположительную активность против патогенных *Staphylococcus aureus* и *Enterococcus faecalis* штаммов. Учитывая активность штамма AZ-130, который продуцирует антибактериальное соединение AZ-130, он был выбран для более детального изучения его характеристик. Основной целью данной работы являлась оптимизация условий выращивания штамма AZ-130, т.е. определение оптимальной среды, температуры инкубации и времени культивирования, при которых синтез антимикробного соединения достигает наивысших значений. Для достижения поставленной цели были протестированы 16 различных условий культивирования (4 разных типа среды при 4 разных температурах). Супернатант клеточной культуры собирали и очищали в 1-й, 2-й и 3-й/5-й дни. Очищенные супернатанты анализировали методом подавления роста и микроразведения в бульоне. Согласно полученным результатам, штамм AZ -130 продуцирует наибольшее количество антимикробного соединения AZ -130, в условиях. когда культивирование осуществляется в течение 2-х дней при температуре 32°C на среде TB с добавлением 2% глюкозы.

Ключевые слова: *Антимикробная активность, антибиотики, биоактивные молекулы, условия культивирования, оптимизация среды, натуральные продукты, первичный метаболит, вторичный метаболит, патогенные бактерии*